

WHAT IS CLAIMED IS:

1                   1.     An *in vivo* method of affinity maturation by competitive activation to  
2 obtain a binding molecule that has an enhanced affinity for a target binding ensemble  
3 member relative to that of a reference binding molecule, the method comprising:

4                   (a) recombinantly altering a population of host cells by  
5                   (i) introducing into the host cells a library of genes encoding candidate  
6 binding molecules;

7                   (ii) introducing into the host cells a competitive activation system  
8 comprising a nucleic acid encoding a responder molecule linked to the target binding  
9 ensemble member, and a nucleic acid encoding a competitor binding molecule linked to an  
10 inhibitor of the responder complex;

11                  (b) incubating the host cells under conditions in which the library and  
12 competitive activation system are expressed and where the responder molecule is activated  
13 when a candidate binding molecule binds to the target binding ensemble member; and

14                  (c) detecting cells having a signal from the responder molecule that  
15 corresponds to a candidate binding molecule binding affinity for the target binding ensemble  
16 member that is greater than that of the reference binding molecule, thereby identifying a  
17 candidate binding molecule with an enhanced affinity for the target binding ensemble  
18 member.

1                   2.     The method of claim 1, wherein the reference binding molecule is a  
2 reference antibody and the target binding ensemble member is an antigen to which the  
3 reference antibody specifically binds.

1                   3.     The method of claim 2, further wherein the competitor binding  
2 molecule is the reference antibody.

1                   4.     The method of claim 3, wherein the reference antibody is an Fab  
2 fragment.

1                   5.     The method of claim 3, wherein the reference antibody is a single  
2 chain Fv.

1                   6.     The method of claim 2, further wherein the candidate binding  
2 molecules are single chain Fvs.

- 1                    7.        The method of claim 2, further wherein the candidate binding  
2 molecules are Fab fragments.
- 1                    8.        The method of claim 2, further wherein the candidate binding  
2 molecules are single V-region domains.
- 1                    9.        The method of claim 1, wherein the candidate binding molecules are  
2 scaffolded peptides.
- 1                    10.       The method of claim 1, wherein the candidate binding molecules are  
2 mutagenized natural ligands of the target binding ensemble member.
- 1                    11.       The method of claim 2, further wherein the library of candidate  
2 binding molecules comprises hybrid antibodies that have at least one CDR in a V<sub>H</sub> or V<sub>L</sub> that  
3 is different from the reference antibody and is from a natural antibody repertoire.
- 1                    12.       The method of claim 11, wherein the hybrid antibodies have either a  
2 V<sub>H</sub> or V<sub>L</sub> from the reference antibody and the corresponding V<sub>H</sub> or V<sub>L</sub> from a natural  
3 antibody repertoire.
- 1                    13.       The method of claim 2, further wherein the competitor binding  
2 molecule is a nonhuman antibody and the candidate binding molecules are antibodies having  
3 at least one human variable region.
- 1                    14.       The method of claim 2, further wherein the competitor binding  
2 molecule is a natural ligand of the antigen that competes with the reference antibody for  
3 binding to the antigen.
- 1                    15.       The method of claim 2, wherein the competitor binding molecule is an  
2 artificial non-antibody ligand of the antigen that competes with the reference antibody for  
3 binding to the antigen.
- 1                    16.       The method of claim 1, wherein the responder molecule is an enzyme.
- 1                    17.       An *in vivo* method of affinity maturation by competitive activation to  
2 obtain a binding molecule that has an enhanced affinity for a target binding ensemble  
3 member relative to that of a reference binding molecule, the method comprising:

4 (a) recombinantly altering a population of host cells by  
5 (i) introducing into the host cells a library of genes encoding candidate  
6 binding molecules;  
7 (ii) introducing into the host cells a competitive activation system  
8 comprising a nucleic acid encoding a responder molecule linked to a competitor binding  
9 molecule, and a nucleic acid encoding an inhibitor linked to the target binding ensemble  
10 member;  
11 (b) incubating the host cells under conditions in which the library and  
12 competitive activation system are expressed and where the responder molecule is activated  
13 when a candidate binding molecule binds to the target binding ensemble member; and  
14 (c) detecting cells having a signal from the responder molecule that  
15 corresponds to a candidate binding molecule affinity for the target ensemble member that is  
16 greater than that of the reference binding molecule, thereby identifying a candidate binding  
17 molecule with an enhanced affinity for the target binding ensemble member.

1 18. The method of claim 17, wherein the reference binding molecule is a  
2 reference antibody and the target binding ensemble member is an antigen to which the  
3 reference antibody specifically binds.

1 19. The method of claim 18, further wherein the competitor binding  
2 molecule is the reference antibody.

1 20. The method of claim 19, wherein the reference antibody is an Fab  
2 fragment.

1 21. The method of claim 19, wherein the reference antibody is a single  
2 chain Fv.

1 22. The method of claim 18, further wherein the candidate binding  
2 molecules are single chain Fvs.

1 23. The method of claim 18, further wherein the candidate binding  
2 molecules are Fab fragments.

1 24. The method of claim 18, further wherein the candidate binding  
2 molecules are single V-region domains.

1                   25.     The method of claim 17, further wherein the candidate binding  
2 molecules are scaffolded peptides.

1                   26.     The method of claim 17, further wherein the candidate binding  
2 molecules are mutagenized natural ligands of the target binding ensemble member.

1                   27.     The method of claim 18, further wherein the library of candidate  
2 binding molecules comprises hybrid antibodies that have at least one CDR in a V<sub>H</sub> or V<sub>L</sub> that  
3 is different from the reference antibody and is from a natural antibody repertoire.

1                   28.     The method of claim 27, wherein the hybrid antibodies have either a  
2 V<sub>H</sub> or V<sub>L</sub> from the reference antibody and the corresponding V<sub>H</sub> or V<sub>L</sub> from a natural  
3 antibody repertoire.

1                   29.     The method of claim 18, further wherein the competitor binding  
2 molecule is a nonhuman antibody and the candidate binding molecules are antibodies having  
3 at least one human variable region.

1                   30.     The method of claim 18, further wherein the competitor binding  
2 molecule is an artificial non antibody ligand of the antigen that competes with the reference  
3 antibody for binding to the antigen.

1                   31.     The method of claim 18, wherein the competitor binding molecule is  
2 an artificial non-antibody ligand of the target antigen that competes with the reference  
3 antibody for binding to the target antigen.

1                   32.     The method of claim 18, wherein the responder molecule is an  
2 enzyme.

1                   33.     An *in vivo* method of affinity maturation by auto-inhibited reactivation  
2 to obtain a binding molecule that has an enhanced affinity for a target binding ensemble  
3 member relative to a reference binding molecule, the method comprising:  
4                   (a) recombinantly altering a population of host cells by  
5                         (i) introducing into the host cells a competitor that binds to the target  
6 binding ensemble member with the same specificity as a reference binding molecule;

7 (ii) introducing into the host cells a nucleic acid encoding a reactivator  
8 complex comprising a reactivator molecule linked to the target binding ensemble member;

9 (iii) introducing into the host cells a library of genes, each of which  
10 encodes an auto-inhibited responder complex comprising a responder molecule linked to an  
11 inhibitor and linked to a candidate binding molecule;

12 (b) incubating the host cells under conditions in which the competitor, the  
13 reactivator complex, and the auto-inhibited responder library are expressed where the  
14 responder molecule is activated when a candidate binding molecule binds to the target  
15 binding ensemble member; and

16 (c) detecting cells having a signal from the responder molecule that  
17 corresponds to a candidate binding molecule affinity for the target binding ensemble member  
18 that is greater than that of the reference binding molecule, thereby identifying a candidate  
19 binding molecule with an enhanced affinity for the target binding ensemble member.

1 34. The method of claim 33, wherein the reference binding molecule is an  
2 antibody and the target binding ensemble member is an antigen to which the reference  
3 antibody specifically binds.

1 35. The method of claim 34, further wherein the competitor is the  
2 reference antibody.

1 36. The method of claim 35, further wherein the reference antibody is an  
2 Fab fragment.

1 37. The method of claim 35, further wherein the reference antibody is a  
2 single chain Fv (scFv).

1 38. The method of claim 34, further wherein the candidate binding  
2 molecules are single chain Fvs.

1 39. The method of claim 34, further wherein the candidate binding  
2 molecules are Fab fragments.

1 40. The method of claim 34, further wherein the candidate binding  
2 molecules are single V-region domains.

- 1                   41.     The method of claim 33, wherein the candidate binding molecules are  
2 scaffolded peptides.
- 1                   42.     The method of claim 33, wherein the candidate binding molecules are  
2 mutagenized ligands.
- 1                   43.     The method of claim 34, further wherein the candidate binding  
2 molecules are hybrid antibodies that have at least one CDR in a V<sub>H</sub> or V<sub>L</sub> that is different  
3 from the reference antibody and is from a natural antibody repertoire.
- 1                   44.     The method of claim 43, wherein the hybrid antibodies have either a  
2 V<sub>H</sub> or V<sub>L</sub> from the reference antibody and the corresponding V<sub>H</sub> or V<sub>L</sub> from a natural  
3 antibody repertoire.
- 1                   45.     The method of claim 34, further wherein the competitor is a nonhuman  
2 antibody and the candidate binding molecules comprise antibodies having at least one human  
3 variable region.
- 1                   46.     The method of claim 34, further wherein the competitor is a scaffolded  
2 peptide that competes with the reference antibody for binding to the antigen.
- 1                   47.     The method of claim 34, further wherein the competitor is an artificial  
2 non-antibody ligand of the antigen that competes with the reference antibody for binding to  
3 the antigen.
- 1                   48.     A method of affinity maturation by self-inhibited reactivation to obtain  
2 a binding molecule that has a higher affinity for a target binding ensemble member than that  
3 of a reference binding molecule, the method comprising:  
4                   (a) recombinantly altering a population of host cells by  
5                         (i) introducing into the host cells a competitor binding molecule that  
6 binds to a target binding ensemble member with the same specificity as the reference binding  
7 molecule,  
8                         (ii) introducing into the host cells a nucleic acid encoding an auto-  
9 inhibited responder complex comprising a responder molecule linked to an inhibitor and to  
10 the target binding ensemble member,

11 (iii) introducing into the host cells a library of genes, each encoding a  
12 reactivator complex, wherein each gene encodes a reactivator molecule linked to a candidate  
13 binding molecule;

14 (b) incubating the host cells under conditions in which the competitor, the  
15 auto-inhibited responder-target binding ensemble member complex, and the reactivator  
16 library complex are expressed and where the responder molecule is activated when a  
17 candidate binding molecule binds to the target binding ensemble member; and

18 (c) detecting cells having a signal from the responder molecule that  
19 corresponds to a candidate binding molecule affinity for the target binding ensemble member  
20 that is greater than that of the reference binding molecule, thereby identifying a candidate  
21 binding molecule with an enhanced affinity for the target binding ensemble member.

1 49. The method of claim 47, wherein the reference binding molecule is a  
2 reference antibody and the target binding ensemble member is an antigen to which the  
3 reference antibody specifically binds.

1 50. The method of claim 49, further wherein the competitor is the  
2 reference antibody.

1 51. The method of claim 49, wherein the reference antibody is an Fab  
2 fragment.

1 52. The method of claim 49, wherein the reference antibody is a single  
2 chain Fv (scFv).

1 53. The method of claim 49, further wherein the candidate binding  
2 molecules are single chain Fvs.

1 54. The method of claim 49, wherein the candidate binding molecules are  
2 Fab fragments.

1 55. The method of claim 49, wherein the candidate binding molecules are  
2 single V-region domains.

1 56. The method of claim 47, wherein the candidate binding molecules are  
2 scaffolded peptides.

1                    57.     The method of claim 47, wherein the candidate binding molecules are  
2     mutagenized natural ligands that specifically bind the target binding ensemble member.

1                    58.     The method of claim 49, further wherein the candidate binding  
2     molecules are hybrid antibodies that have at least one CDR in a V<sub>H</sub> or V<sub>L</sub> that is different  
3     from the reference antibody and is from a natural antibody repertoire.

1                    59.     The method of claim 58, wherein the hybrid antibodies have either a  
2     V<sub>H</sub> or V<sub>L</sub> from the reference antibody and the corresponding V<sub>H</sub> or V<sub>L</sub> from a natural  
3     antibody repertoire.

1                    60.     The method of claim 49, further wherein the reference antibody is a  
2     nonhuman antibody and the candidate binding molecules are antibodies having at least one  
3     human variable region.

1                    61.     The method of claim 49, further wherein the competitor is a natural  
2     ligand of the target antigen that competes with the reference antibody for binding to the  
3     antigen.

1                    62.     The method of claim 49, wherein the competitor is a natural ligand of  
2     the antigen that competes with the reference antibody for binding to the antigen.